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An ancient role for Gata-1/2/3 and Scl transcription factor homologs in the development of immunocytes

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ABSTRACT

Although vertebrate hematopoiesis is the focus of intense study, immunocyte development is well-characterized in only a few invertebrate groups. The sea urchin embryo provides a morphologically simple model for immune cell development in an organism that is phylogenetically allied to vertebrates. Larval immunocytes, including pigment cells and several blastocoelar cell subtypes, emerge from a population of non-skeletal mesodermal (NSM) precursors that is specified at the blastula stage. This ring of cells is first partitioned into oral and aboral fields with distinct blastocoelar and pigment cell gene regulatory programs. The oral field is subsequently specified into several distinct immune and non-immune cell types during gastrulation. Here we characterize the oral NSM expression and downstream function of two homologs of key vertebrate hematopoietic transcription factors: *SpGatac*, an ortholog of vertebrate *Gata-1/2/3* and *SpScl*, an ortholog of *Scl/Tal-2/Lyl-1*. Perturbation of SpGatac affects blastocoelar cell migration at gastrulation and later expression of immune effector genes, whereas interference with SpScl function disrupts segregation of pigment and blastocoelar cell precursors. Homologs of several transcription regulators that interact with Gata-1/2/3 and Scl factors in vertebrate hematopoiesis are also co-expressed in the oral NSM, including *SpE-protein*, the sea urchin homolog of vertebrate *E2A/HEB/E2-2* and *SpLmo2*, an ortholog of a dedicated cofactor of the Scl–GATA transcription complex. Regulatory analysis of *SpGatac* indicates that oral NSM identity is directly suppressed in presumptive pigment cells by the transcription factor SpGcm. These findings provide part of a comparative basis to understand the evolutionary origins and regulatory biology of deuterostome immune cell differentiation in the context of a tractable gene regulatory network model.

Introduction

Specialized immune cells that guard against pathogens, regulate symbiosis and maintain tissue homeostasis are nearly ubiquitous among bilaterian animals. General features of these cell systems, such as division into phagocytic and granular subsets, are also widespread (Hartenstein, 2006), yet little is known of how these cells and their associated genetic circuitry are related across phyla. Immune recognition and effector mechanisms evolve rapidly (Clark et al., 2007; Hughes and Friedman, 2008) and are therefore problematic as comparative tools. Instead, a more fruitful approach to characterizing these cellular relationships may come from comparative investigations of developmental gene regulatory networks. These may contain conserved core elements of circuitry that can lend further insight to this problem (Davidson and Erwin, 2006).

The sea urchin embryo provides a powerful model for this purpose. It is well suited for characterizing developmental gene regulatory networks (Oliveri et al., 2008). Further, echinoderm larvae were the focus of classical studies that defined animal cellular immunity (Metchnikoff, 1891) and several recent studies have addressed immune functions in the larva (Furukawa et al., 2009; Silva, 2000; Yang et al., 2010). Our investigations in the

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purple sea urchin, *Strongylocentrotus purpuratus*, indicate that the feeding larva is equipped with a well developed immune system. We have identified an expansive suite of immune receptors and effectors that are expressed in the larva and have characterized several distinct immune cell types on the basis of cell behavior and gene expression (Buckley and Rast, 2012; Hibino et al., 2006; E.C.H.H. and J.P.R., unpublished).

Larval immunocytes develop from two major derivatives of the embryonic non-skeletal mesoderm (NSM), also known as secondary mesenchyme cells (SMCs) because of their mesenchymal phenotype. The first of these, the pigment cells, emerges in early gastrulation and quickly differentiates into a uniform cell type (Gibson and Burke, 1985, 1987; Kominami, 2000a, 2000b; Ransick and Davidson, 2006). These cells are highly motile, engage in continuous surveillance-like activity and migrate to sites of bacterial infection (E.C.H.H. and J.P.R, unpublished). The second category of cells that contribute to larval immunity is collectively known as blastocoelar cells. These cells ingress from the tip of the archenteron about ten hours after pigment cells delaminate (Tamboline and Burke, 1992) and differentiate into a heterogeneous assemblage of cells with immune and non-immune functions by the onset of feeding (Hibino et al., 2006; Katow et al., 2004). Several of these blastocoelar cell types display immune-like motile behavior, are capable of efficient phagocytosis, and express immune effector genes (Hibino et al., 2006).

A series of regulatory events leading to the specification and initial subdivision of the NSM is well-characterized. The NSM originates from a ring of blastomeres that lie in direct contact with the skeletal mesoderm (SM). Notch signaling is required for specification of all NSM-derived cell types including pigment and blastocoelar cells (Sherwood and McClay, 1999). A Delta ligand expressed by the SM provides the source of this signal (Sweet et al., 2002). One of the first regulatory state changes that differentiates the NSM ring is the expression of the transcription factor glial cells missing (*SpGcm*) in a one-cell thick ring surrounding the skeletogenic precursors in the early blastula (Ransick et al., 2002). *SpGcm* is a direct target of Delta–Notch (D/N) signaling (Ransick and Davidson, 2006) and the timing of this D/N signal has been thoroughly characterized with respect to the early specification of the NSM relative to surrounding endoderm (Croce and McClay, 2010). A comprehensive study of downstream D/N signal targets in the NSM (Materna and Davidson, 2012) identifies *SpGcm* as the first spatially localized transcription factor activated by the D/N signal in the NSM lineage and the presence of a feed-forward circuit involving the transcription factor SpGataE, which at this stage shows an identical expression pattern in all the NSM cells.

At the mid-blastula stage, a set of pigment cell-specific genes are activated in the entire NSM ring (Calestani et al., 2003; Calestani and Rogers, 2010; Ransick and Davidson, 2006, 2012; Ransick et al., 2002). At the mesenchyme blastula stage, following ingression of the skeletogenic lineage, the NSM ring is subdivided into several regions that were initially characterized in cell fate mapping experiments (Ruffins and Ettensohn, 1993, 1996). Just prior to the ingression of the skeletogenic mesenchyme, the precursors of the blastocoelar cells, which are located on the oral side of the NSM ring, terminate expression of *SpGcm* and other pigment cell-specific genes (Calestani et al., 2003; Ransick et al., 2002) and begin to express a suite of oral NSM transcription factors (Materna et al., 2013). This oral–aboral division of the NSM is downstream of Nodal signaling (Duboc et al., 2010) and it is mediated by the expression of the *SpNot* gene which extends into the oral side of the NSM (Materna et al., 2013). Interestingly, perturbation analysis shows that at mesenchyme blastula *SpNot* expression is required for both up-regulation of oral NSM genes and down-regulation of aboral NSM genes. At this developmental stage the gene encoding the Delta ligand is the only characterized regulatory gene that remains expressed in the entire NSM lineage.

Here we are interested in the gene regulatory network that lies downstream of the initiation of the oral NSM regulatory program and, ultimately, how this transcriptional program contributes to the development of blastocoelar immune cell subtypes. Hence, we characterize the expression and downstream function of the sea urchin homologs of *Gata-1/2/3*, *SpGatac*, and of *Scl/Tal-2/Lyl-1*, *SpScl*, in the context of NSM and the development of larval immunocytes. Homologs of these genes are also important regulators of hematopoiesis in vertebrates (Anderson, 2006; Galloway et al., 2008; Laslo et al., 2008; Maeno, 2003). Both *SpGatac* and *SpScl*, along with other hematopoietic transcription factors, are transiently expressed in a spatial pattern consistent with a role in blastocoelar cell specification. Perturbation of these factors disrupts NSM patterning and immunocyte development. Perturbation of SpGatac results in an apparent defect of mesenchymal cell motility, whereas SpScl perturbation disrupts partitioning of the NSM. A negative feedback mechanism is also evident that blocks *SpGatac* expression in differentiating pigment cells.

Materials and methods

Animals and embryo culture

S. purpuratus specimens were obtained from Westwind Sealab Supplies, Victoria, BC, Canada and Point Loma Marine Invertebrate Lab, Lakeside, CA, USA. Adults were maintained in aquaria in artificial sea water (Instant Ocean) at 13 °C. Fertilized embryos were kept in 0.45 μM filtered Instant Ocean in Petri dishes or stirred cell culture flasks at 15 °C.

Reverse transcription quantitative PCR (RT-qPCR)

RT-qPCR analysis was performed as previously described (Fugmann et al., 2006; Rast et al., 2002). For injected embryos, 50–500 embryos were collected per sample and RNA was extracted with an RNeasy Micro kit with on-column DNase treatment (Qiagen). cDNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems) with random hexamers. qPCR cycling was performed using SYBR Green PCR master mix (Applied Biosystems). The oligonucleotides that were used to quantify transcripts are listed in Table S1.

For some transcript prevalence measurements, coelomocytes were isolated from two *S. purpuratus* adults by mixing coelomic fluid with an equal volume of calcium–magnesium-free sea water containing 20 mM EDTA. RNA was isolated from cell pellets with Trizol (Invitrogen), DNase treated (DNA-free, Ambion) and used for reverse transcription (Superscript III, Invitrogen). RT-qPCR was carried out as described above.

Rapid amplification of cDNA ends (RACE) and cDNA analysis

Message sequences for *SpGatac* (short transcript) and *SpE-protein* were determined previously from coelomocyte cDNA (Pancer et al., 1999) and verified from embryonic cDNA. *SpScl*, *SpId* and *SpLmo2* were identified through BLAST analysis of the sea urchin genome (Hibino et al., 2006). Full transcript sequences were determined using a RACE strategy (GeneRacer, Invitrogen) using cDNA isolated from the mesenchyme blastula and early gastrula stage embryos. Transcript sequences for *SpScl*, *SpId* and *SpGatac* (long transcript) from our RACE data are consistent with transcriptome sequence available at SpBase (www.spbase.org; Tu et al., 2012). Sequence of the *SpGatac* (short transcript) was

verified by RACE, targeted RT-PCR and is consistent with our larval transcriptome data.

Whole mount in situ hybridization (WMISH) and imaging

Transcript detection was performed as previously described (Minokawa et al., 2004; Ransick et al., 2002). Fluorescent WMISH was carried out according to published protocols (Croce and McClay, 2010). Some images were obtained using the optical sectioning acquisition setting on an Apotome equipped microscope (Zeiss).

Morpholino antisense oligonucleotides (MASO) and reporter constructs

MASO preparations were injected at 150–200 mM as described (Angerer and Angerer, 2004). MASO sequences are as follows: SpGatac translation block, 5′-CATTAAAAGAAAATAACAAGTTCAC-3′; SpGatac splice block, 5′-TAAAATACTTACCTGTTGTTGATC-3′; SpScl, 5′-GGTCAGGAGAAGTGCGATTGGTCCG-3′. Linearized bacterial artificial chromosome (BAC) DNA was injected at 50–100 copies/pl. Linearized plasmid DNA was injected at 500 copies/pl (Rast, 2000).

Preparation of reporter constructs

Introduction of green fluorescent protein (GFP) coding sequence into the BAC vectors containing *SpGatac* (BAC 81C18) and *SpScl* (BAC 54D1) was carried out by homologous recombination in EL250 *Escherichia coli* cells (Copeland et al., 2001). DNA from BAC reporter constructs was purified on CsCl gradients and linearized with AscI (New England Biolabs). MASO-resistant *SpGatac* and *SpScl* BAC constructs were engineered by substituting the 25 nucleotide MASO binding site with the corresponding upstream sequence from *SpEndo16* (antisense terminating at the ATG start, see supplementary figures), by oligonucleotide driven homologous recombination in EL250 cells (Swaminathan et al., 2001).

The 5 kb GFP reporter construct for *SpGatac* was amplified from the 81C18 GFP BAC, using Expand Long Template polymerase (Roche), cloned into the pGEM-T Easy vector (Promega). Mutagenesis of the potential SpGcm binding sites was performed by overlap PCR and subcloning of mutated fragments into the unmutated 5 kb GFP construct. Potential Gcm binding sites were chosen based on a high enrichment score (Berger and Bulyk, 2009; Berger et al., 2006) and conservation with homologous sequence from *Lytechinus variegatus* (Fig. S6). Mutations were confirmed by sequencing.

Results

Homologs of vertebrate Gata1/2/3, Scl and associated transcription factors are expressed in larval immunocyte development

The gene encoding *SpGatac* is expressed in the embryo and larva as two alternatively spliced isoforms. The short form (*SpGatac*) is identical to that isolated from coelomocytes (Pancer et al., 1999) and encodes a 431 amino acid protein. The longer form (*SpGatac-L*) encodes a 583 amino acid protein that incorporates two additional upstream exons spliced internally into the *SpGatac* first exon (Fig S1A). The short *SpGatac* transcript greatly predominates during the embryonic stages investigated here (expressed more than 40-fold higher than *SpGatac-L* until early pluteus stage) whereas expression of *SpGatac-L* is elevated only later in larval development (C.S.S. and J.P.R., unpublished). Importantly, the phenotype of antisense perturbation blocking splicing of both forms is indistinguishable from that targeted to the translation start of the short form in the blastula, gastrula and prism stages (see below). Given this expression profile we focus on the short *SpGatac* form. A single *SpScl* transcript is evident from RACE derived sequences using RNA from the embryo and pluteus stages. This SpScl protein is encoded in one exon with a separate noncoding exon containing a very long (763 nt) 5′ UTR (Fig. S1A).

Time course profiles for expression of *SpGatac*, *SpScl* and several interacting transcription factors (*SpE-protein*, *SpId* and *SpLmo2*) are shown in Fig. 1 (A and B). *SpGatac* expression is initially evident at 18–20 h post-fertilization (hpf) and peaks in the mesenchyme blastula and early gastrula stage embryo (24–33 hpf). *SpScl* has a similar profile in these stages, although a level of earlier expression that could not be localized is also evident at cleavage stage and in the early blastula. At mesenchyme blastula (24 hpf) the NSM ring is divided into an aboral region containing the pigment cell precursors and an oral sector from which the blastocoelar cells emerge (Ransick et al., 2002; Ruffins and Ettensohn, 1996; Fig. 1C). WMISH co-localizes expression of *SpGatac* and *SpScl* to a subset of mesodermal cells (Fig. 1D–K). Both genes are expressed in a discrete patch of cells in the NSM at mesenchyme blastula stage (24 hpf; Fig. 1D and G). In the few hours preceding this tightly localized expression pattern, the spatial distribution of lower level expression is variable among embryos within the oral–aboral axis of the NSM ring and is sometimes evident in one or two cells outside of the NSM (Fig S2). This expression pattern is increasingly resolved during the period of 22–24 hpf, such that by 24 hpf all embryos exhibit consistent expression in a patch of 10–14 oral NSM cells. Co-staining with the aboral presumptive pigment cell marker *SpGcm* at 24 hpf shows that expression is excluded from the pigment cell domain, although limited overlap sometimes occurs at the border (Fig. 1J and K). Two-color fluorescent WMISH targeting *SpGatac* and *SpScl* shows that expression of these transcription factors completely overlaps at this stage (not shown). Elevated expression of these genes continues as the archenteron elongates. As blastocoelar cells ingress (40–48 hpf), both *SpGatac* and *SpScl* are down-regulated such that low expression is evident at the archenteron tip and in ingressing cells (black arrowheads, Fig. 1E and H) but rarely seen in cells already in the blastocoel. At late gastrula stage, *SpGatac* and *SpScl* expression is also evident in a subset of four to six aboral skeletogenic mesenchyme cells (48 hpf; arrows Fig. 1E and H). This expression pattern persists through the early pluteus stage (72 hpf) when transcripts are also detected in cells of one or both coelomic pouches (white arrowheads, Fig. 1F and I). By 96 hpf, expression of both *SpGatac* and *SpScl* is usually restricted to the right coelomic pouch and aboral skeletal cells (data not shown).

To characterize spatial expression by an alternative means, we constructed *SpGatac*-BAC GFP (short transcript) and *SpScl*-BAC GFP reporters. These recapitulate both the mesenchyme blastula expression phase in the NSM, and later skeletal and coelomic pouch cell expression (Fig. S1B–G). Notably GFP fluorescence from the reporter is not attenuated upon blastocoelar cell ingression as is observed for mRNA in WMISH. This may be the result of a prolonged half-life of GFP in these cells or may suggest that low levels of expression persist after ingression. Due to mosaic integration of the transgene, only a fraction of blastocoelar cells are expected to display GFP expression. Because a subset of the blastocoelar cells and the skeletal mesenchyme form separate syncytial cell networks, GFP expressed from the reporter is observed in cells throughout the blastocoel in the late gastrula and pluteus, although many blastocoelar cells remain negative.

In vertebrates, Gata-1/2/3 and Scl/Tal-1 factors are well-known to regulate hematopoietic genes by binding to DNA target sites in conjunction with several partner transcription factors and cofactors. Therefore we also characterized expression of homologs of factors known to interact with this system. SpE-protein (formerly

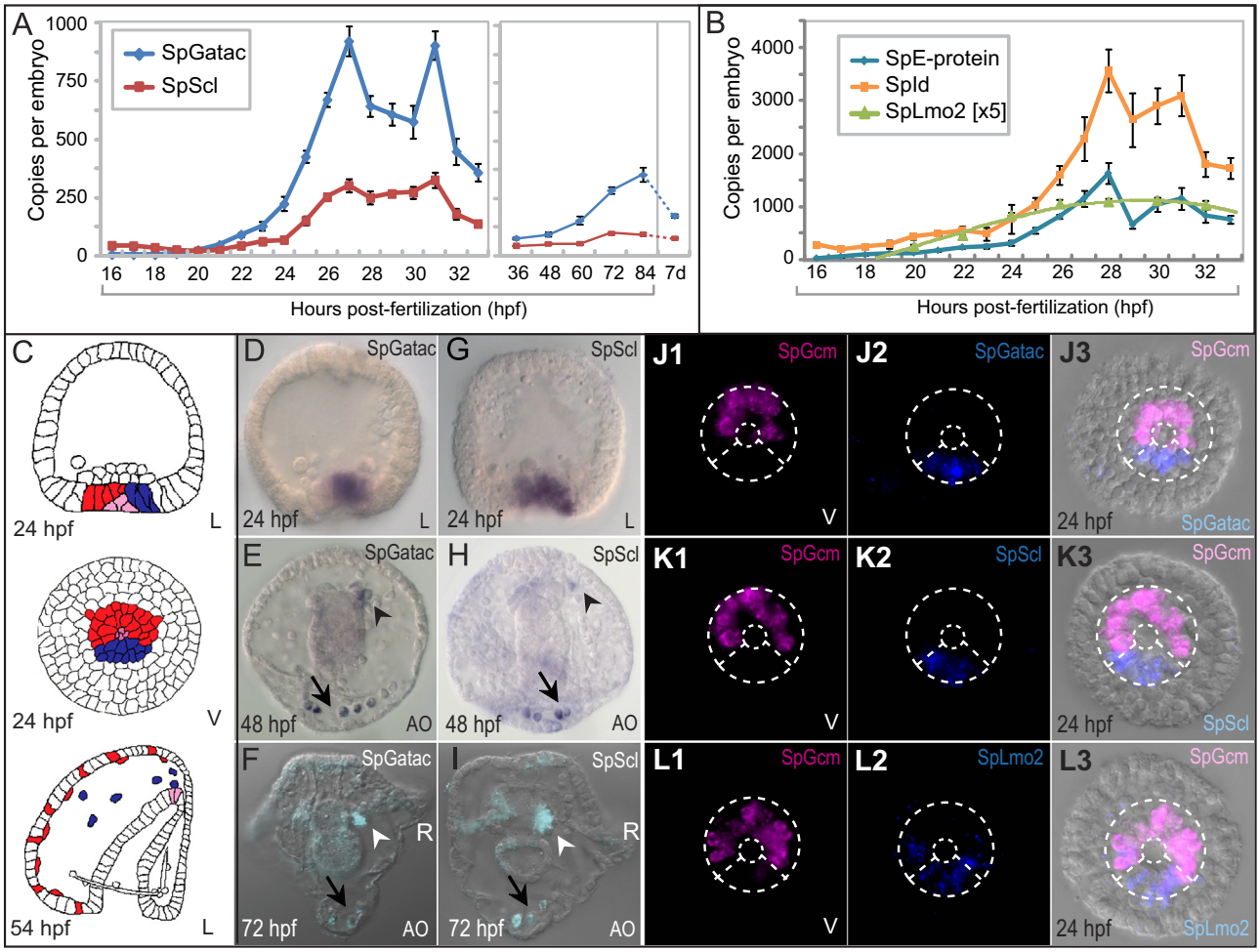


Fig. 1. Expression of *SpGatac*, *SpScl* and associated transcription factors and cofactors. (A) *SpGatac* and *SpScl* transcript prevalence throughout embryonic development as measured by RT-qPCR. Estimate of copies per embryo is set to probe excess measurement of *SpGatac* at peak. (B) Prevalence measurements for factors that are known to interact with *Gata-1/2/3* and *Scl* homologs in vertebrates. *SpLmo2* transcript prevalence measurements are scaled up by a factor of five to allow visualization. Error bars in A and B represent standard deviation among replicates. (C) Illustration of NSM regulatory territories at mesenchyme blastula stage (24 hpf) and the position of their progeny in the 54 h late gastrula embryo. Blue, oral blastocoelar cell precursors; red, aboral pigment cell precursors; pink, small micromere descendants. (D–I) *In situ* hybridization localizes *SpGatac* and *SpScl* expression to a subset of the NSM in the mesenchyme blastula (D and G), to the delaminating secondary mesenchyme cells (black arrowheads) and aboral skeletal mesenchyme (arrow) in the late gastrula (E and H) and to the aboral skeletal mesenchyme (arrow) and primarily right coelomic pouch cells (white arrowheads) in early pluteus stage embryos (F and I). (J and K) Two color fluorescent *in situ* hybridization co-staining with *SpGcm* as a marker for aboral pigment cell precursors localizes *SpGatac* (J) and *SpScl* (K) to the oral NSM at 24 hpf. (L) Co-staining with *SpGcm* localizes expression of the transcription cofactor *SpLmo2* to the oral NSM at 24 hpf. V, vegetal view with oral side to the bottom; L, lateral view with oral side to the right; AO, aboral view; R, right side.

described as SpE2A, Hibino et al., 2006), the sea urchin homolog of vertebrate E2A/HEB/E2-2, is the predicted heterodimeric partner of SpScl in the NSM. The temporal expression profile of *SpE-protein* coincides with the period of oral/aboral NSM segregation (Fig. 1B). WMISH shows that *SpE-protein* is expressed ubiquitously at the mesenchyme blastula stage (Fig. S3A), although alternative splice forms are differentially regulated in the oral–aboral NSM (C.M.S., C.S.S. and J.P.R., unpublished). Expression of *SpId*, the sea urchin homolog of the vertebrate *Id1/2/3/4* paralogs, is also expressed throughout the embryo in the mesenchyme blastula and gastrula (Fig. 1B), but at the mesenchyme blastula stage its expression level is elevated in the aboral NSM pigment cell precursors (Fig. S3B). Thus SpId is positioned to participate along with SpScl in modulating SpE-protein activity across the oral–aboral NSM.

SpLmo2 is a homolog of vertebrate Lmo2 which acts as a transcription cofactor bridging Gata and Scl proteins when they bind cooperatively at DNA target sites. *SpLmo2* expression levels, while low, reach a broad maximum at around 27–28 hpf, as *SpGatac* and *SpScl* are also at their highest (Fig. 1B). Expression was detected in the oral NSM (Fig. 1L), which coincides with *SpScl* and *SpGatac* although it may be expressed somewhat more widely. This is consistent with expression of the homolog of *Lmo2* in *Paracentrotus lividus* (Duboc et al., 2010). Collectively these data show that by mesenchyme blastula stage the blastocoelar cell precursors have a complex and distinct regulatory state that shares important transcription factors homologs with the regulatory program of vertebrate hematopoiesis.

SpGatac perturbation impedes blastocoelar cell ingression and immune cell differentiation

To further characterize the role of SpGatac in blastocoelar cell development, we perturbed its function by MASO injection. To track *SpGatac* expressing cells in the context of the perturbed background, we co-injected an *SpGatac*-GFP BAC reporter bearing a mutated MASO binding region (Fig. S4). The MASO-resistant version of the *SpGatac* GFP BAC is expressed identically to the unmodified form (not shown). Translational interference of *SpGatac* results in a delay or block in archenteron elongation

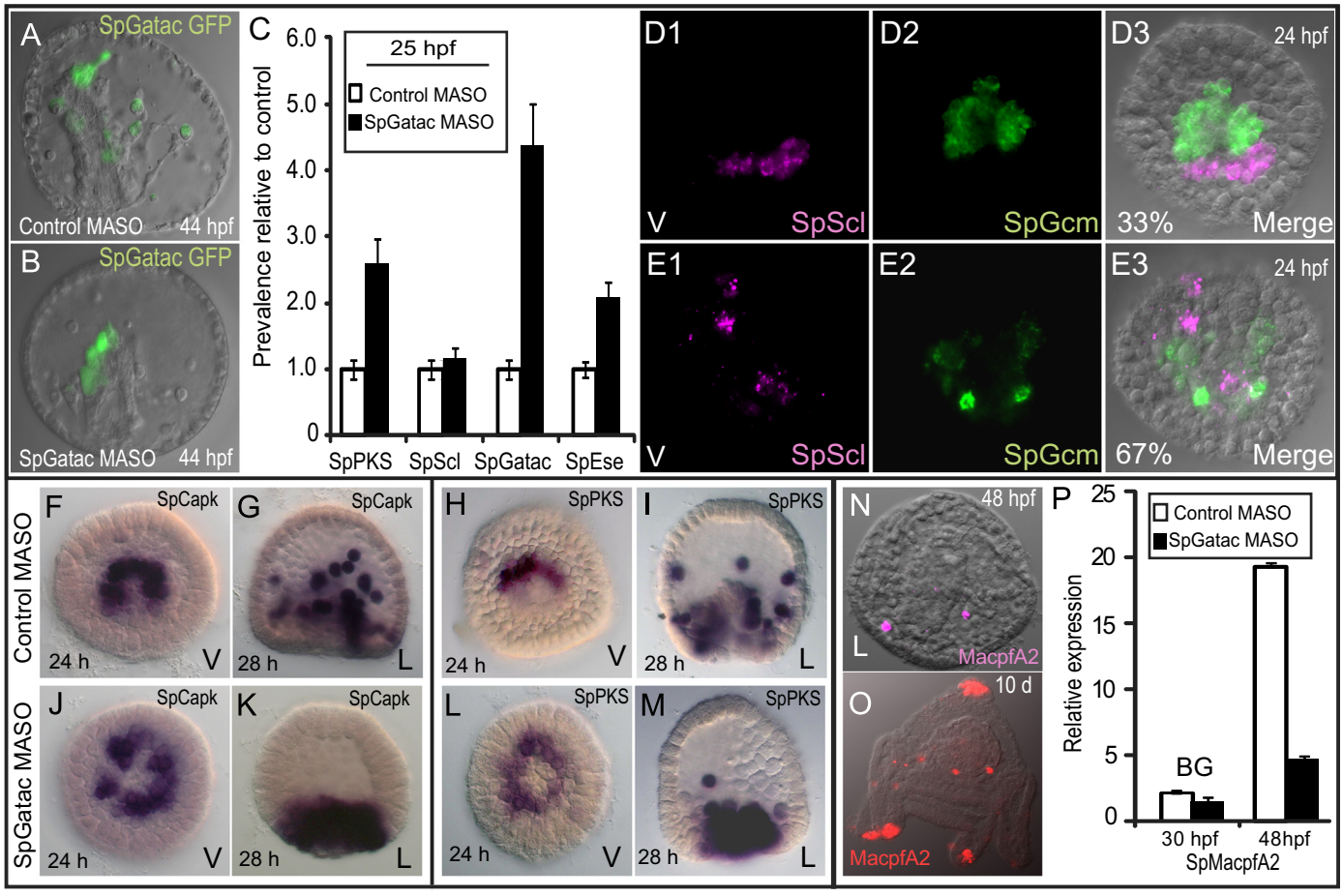


Fig. 2. Perturbation of SpGatac function interferes with NSM gene expression and later ingression. (A and B) Zygotes were co-injected with SpGatac or control MASO and a MASO-resistant *SpGatac* BAC-based GFP reporter (Fig. S4). Embryos co-injected with control MASO (A) display normal archenteron elongation and blastocoelar cell ingression as visualized at 44 hpf (gastrula). In embryos co-injected with a SpGatac MASO (B), archenteron elongation is truncated and GFP expressing NSM cells fail to detach from the archenteron tip. (C) Prevalence of NSM gene transcripts in SpGatac MASO injected embryos relative to control measured by RT-qPCR in the mesenchyme blastula. (D and E) Two color fluorescent *in situ* hybridizations with *SpScl* and *SpGcm* probes in SpGatac MASO injected embryos indicates varying degrees of disruption in SpGatac perturbed embryos. Some embryos (33%) display normal expression patterns (D1–3), others (67%) have disrupted expression of both *SpScl* and *SpGcm* (E1–3). Data were replicated in two independent experiments. (F–M) *SpGatac* expression affects pigment cell patterning and migration. Normal aboral patterning and early pigment cell ingression (F-I, control MASO injected) are disrupted in embryos injected with SpGatac MASO (J–M) as indicated by *in situ* hybridization with probes to two pigment cell markers, *SpCapk* (F, G, J, and K) and *SpPKS* (H, I, L, and M). (N–P) SpGatac disruption affects later expression of an immune marker in a blastocoelar cell subtype. *SpMacpfA2* is normally expressed in two blastocoelar cells starting just after ingression (N) and in a larger number of globular cells in the feeding larva (O). SpGatac MASO injection decreases expression of SpMacpfA2 relative to control MASO injection as measured by RT-qPCR (P). At 30 hpf *SpMacpfA2* expression is near background (BG) and undetectable by WMISH. V, vegetal view; L, lateral view.

and blastocoelar cell ingression (Fig. 2A and B). When the archenteron does form, it is truncated or thin. This is consistent with the contribution of NSM cells to archenteron elongation (Dan and Okazaki, 1956; Hardin, 1988) although it may derive from other migratory defects or by impairing signals necessary for continued morphogenesis. *SpGatac* MASO injected embryos have few migrating cells in the blastocoel and GFP positive cells fail to delaminate from the tip of the archenteron (Fig. 2A and B). Although they produce filopodia and are motile, these cells appear to remain attached in the epithelial layer (Fig. 2B). Embryos injected with a second MASO that independently targets the first splice acceptor site of *SpGatac* produces an identical phenotype (Fig. S5A–C). We verified the efficiency of the MASO targeting the translation start site by observing its ability to interfere with a transgenic hatching enzyme promoter (*SpHe*)-GFP reporter construct that contained the SpGatac MASO target sequence fused to GFP (Fig. S5D–F).

Embryos injected with the SpGatac MASO display elevated levels of *SpGatac* transcript (Fig. 2C; Table S2). These data suggest that SpGatac negatively regulates its own expression, either directly or indirectly. This is consistent with the presence of a transient peak of expression of *SpGatac* at mesenchyme blastula to early gastrula stages. Expression levels of several other oral NSM transcription factors are not strongly affected by *SpGatac* perturbation when measured by quantitative PCR, although *SpEse*, an Ets factor expressed in the oral NSM (Rizzo et al., 2006) may be somewhat elevated. The pigment cell differentiation gene *polyketide synthase* (*SpPKS*; Calestani et al., 2003) is sometimes elevated in SpGatac MASO injected embryos (Table S2), which is consistent with non-quantitative observations from WMISH where expression of pigment cell genes is enhanced relative to control injected embryos (see below).

Patterning of the oral and aboral NSM expression domains is partially disrupted at mesenchyme blastula stage in *SpGatac* MASO injected embryos (Fig. 2D–M). Expression of *SpScl* was expanded or atypical in 67% of *SpGatac* MASO injected embryos (Fig. 2E), compared to control MASO injected embryos. These data were replicated in an independent experiment. The domain of *SpScl* expression in *SpGatac* perturbed embryos is often expanded but expression appears lower per cell, consistent with similar overall *SpScl* expression levels measured by qPCR (Fig. 2C).

Pigment cell migration is also impaired in SpGatac MASO injected embryos (Fig. 2F–M). Two pigment cell markers (*SpPKS* and *SpCapk*, encoding a cyclic AMP dependent protein kinase) exhibit expanded expression into the oral NSM at 24 hpf (Fig. 2J and L) and initiation of pigment cell ingression is delayed at 28 hpf (Fig. 2K and M) relative to control MASO injected embryos (Fig. 2G and I). Altered expression of both *SpScl* and pigment cell markers upon SpGatac perturbation suggests that disturbance in the oral mesoderm can affect specification throughout the NSM.

We next assessed the effect of SpGatac perturbation on the expression of a marker of immune cell differentiation, *SpMacpfA2*. This gene encodes a perforin-like factor that is specifically and constitutively expressed by a subtype of blastocoelar cell (globular cells). These granular cells exhibit surveillance-like motile behavior in the larva (E.C.H.H. and J.P.R., unpublished). *SpMacpfA2* expression initiates in a small number of cells shortly after ingression from the archenteron tip at late gastrulation (Fig. 2N) and continues in larval globular cells that occupy the blastocoel, arm tips and apex (Fig. 2O). SpGatac MASO perturbation results in a four-fold decrease in *SpMacpfA2* expression as cells begin to strongly express this marker at 48 hpf (Fig. 2P).

We rescued the MASO phenotype by transgenic expression of *SpGatac*. To accomplish this we assembled a BAC-based *SpGatac* transgene that is resistant to the translation interference MASO (Fig. S4). Activity of this BAC transgene was traced with the SpGatac-GFP reporter construct (also resistant to the SpGatac MASO). By co-injecting these constructs along with the SpGatac MASO, we were able to rescue the gastrulation defect in embryos that express GFP and thus have incorporated the BAC-transgenes into the oral NSM domain (Fig. 3A and B). Co-injection of the tracer and MASO with DNA from a unrelated control BAC was unable to rescue the phenotype (Fig. 3C). These experiments demonstrate that rescue in the presence of SpGatac MASO requires both the introduction of the *SpGatac* transgene and proper mosaic expression. Although this type of mosaic rescue strategy may be inadequate for widely expressed genes, for genes like *SpGatac* that are expressed in small domains, rescue of even a subset of cells is sufficient to advance the embryo. This technique additionally provides a new strategy to experimentally introduce modified factors into the embryo in a regulated fashion against a perturbed endogenous background.

SpScl expression is required for normal oral–aboral NSM patterning

Scl and its paralogs are important transcription factors in vertebrate hematopoiesis and often bind DNA target sites cooperatively with members of the hematopoietic subfamily of GATA factors such as Gata-1 and Gata-2 (Tripic et al., 2008; Wozniak et al., 2008). Therefore, we investigated the role of *SpScl* in the NSM developmental program of the sea urchin embryo. MASO perturbation of SpScl results in an increased number of cells within the blastocoel and a reduction or absence of pigment cells, as well as delayed skeletogenic mesenchyme ingression and reduced skeletal rod elongation at prism stage (Fig. 4). This phenotype is unexpected given the expression domain of *SpScl*. While SpScl and other oral transcription factors are sometimes initially expressed variably and transiently at low levels in mesoderm outside of the oral NSM (Fig. S2), stable, elevated expression is restricted to oral NSM by 22–24 hpf in the mesenchyme blastula (Fig. 1K). Low *SpScl* expression is also evident from transcript prevalence measurements in cleavage-stage embryos and early blastulae but localized expression is not evident in WMISH before 18–20 hpf. This implies ubiquitous expression and per cell levels that are below physiologic relevance.

To validate this perturbation, we confirmed the efficiency of the SpScl MASO with an *SpScl*/GFP fusion transgenic reporter construct driven by the *SpHE* promoter (Fig. S5G–I) and verified specificity by rescuing SpScl function in the MASO injected background (Fig. 5) as described above for the SpGatac perturbation. Rescue was assayed by counting *SpPKS* expressing pigment cell precursors by WMISH in 25 hpf embryos. In embryos where mosaic GFP transgene expression was detected in the vegetal plate, the pigment cell precursor number in SpScl MASO co-injected embryos (15.9 ± 0.9) was nearly identical to control MASO co-injected embryos (18.0 ± 1.2) indicating that the SpScl MASO resistant BAC was able to rescue pigment cell patterning. In contrast embryos from the same injection have a greatly reduced number of *SpPKS* positive cells (5.8 ± 1.3) when the rescue construct did not integrate in the appropriate territory of the embryo as indicated by the absence of GFP positive cells. Likewise, embryos that were co-injected with an arbitrary control BAC (*SpRag1/2L*) had few pigment cell precursors (4.5 ± 1.8) as is typical of the SpScl MASO phenotype. This result was obtained

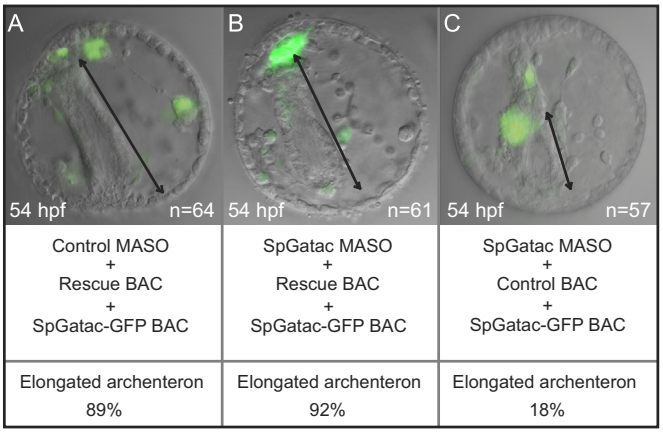
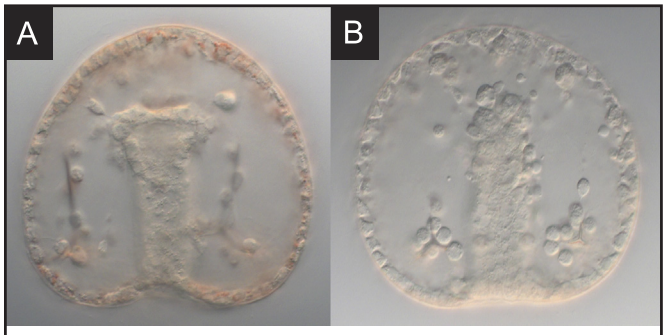


Fig. 3. SpGatac perturbation can be rescued by transgenic introduction of *SpGatac*. Injection of MASO-refractory *SpGatac* BAC mitigates the effect of SpGatac MASO injection. (A and B) Co-injection of the rescue BAC and the MASO-resistant *SpGatac* BAC-GFP reporter with either the control MASO (A) or SpGatac MASO (B) restores archenteron elongation and blastocoelar cell delamination. (C) Co-injection of the SpGatac MASO with an arbitrary control BAC (*SpRag2L* reporter) and GFP reporter, however, fails to rescue the MASO phenotype. The proportion of embryos displaying normal morphology as a percentage of total of GFP positive embryos is indicated for each experimental condition. Double-headed arrows indicate extent of archenteron elongation. Images are representative of the most prevalent phenotype for the given experimental condition. GFP positive fractions ranged from 58% to 64% for each injection, consistent with expectations for mosaic incorporation and with other experiments using the *SpGatac*-GFP BAC in unperturbed embryos.



Phenotype	MASO	
	Control	SpScl
Normal	142	8
Few or no pigment cells/no skeleton	16	147
No archenteron	0	38
Total	158	193

Fig. 4. Perturbation of SpScl affects pigment cell specification. Embryos were injected with control or SpScl MASO and imaged at 58 hpf (late gastrula stage). (A) Control MASO injected embryos develop normally. (B) Most SpScl MASO injected embryos display a reduced number or absence of pigment cells and a failure to elongate skeletal rods. A minority of SpScl perturbed embryos fail to elongate an archenteron. The table tallies the number of embryos that display normal morphology or the abnormalities described above. These numbers are representative of two additional experiments carried out with embryos derived from independent fertilizations.

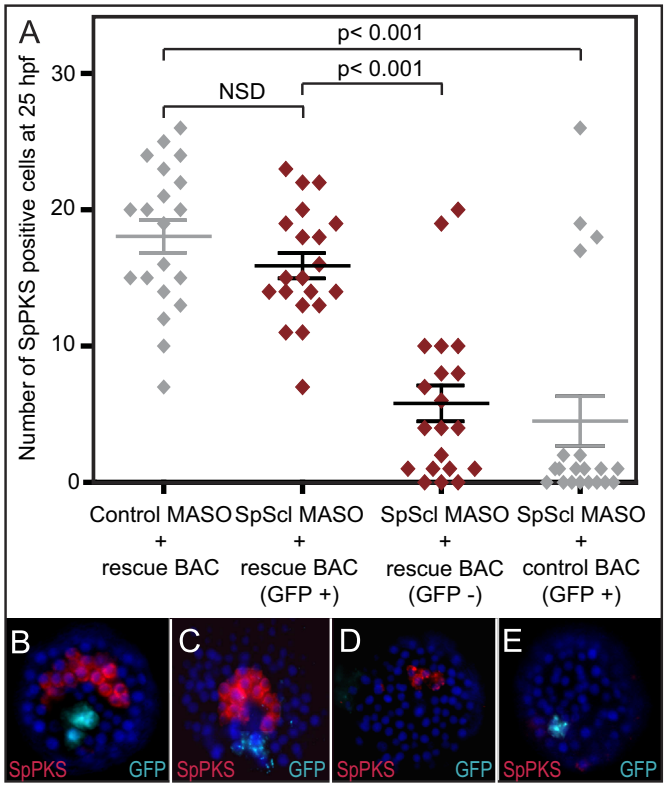


Fig. 5. Regulated transgenic expression of *SpScl* rescues pigment cell specification in SpScl perturbed embryos. (A) A normal number of presumptive pigment cells is restored in SpScl MASO injected embryos after co-injection with an SpScl BAC engineered to be refractory to MASO interference. Cell numbers are determined by counting *SpPKS* positive cells at 25 hpf in WMISH. All treatments were co-injected with a SpScl-GFP BAC construct to mark transgenic expression. For each experimental condition, twenty embryos were scored for the presence of GFP staining in the vegetal plate and the number of *SpPKS* positive pigment cell precursors was counted in two color fluorescent WMISH. (B–E) Representative *in situ* hybridization images that correspond to treatments in the graph above: Control MASO and *SpScl* rescue BAC (B), SpScl MASO and *SpScl* rescue BAC with GFP expression (C), SpScl MASO and *SpScl* rescue BAC without GFP expression (D), and SpScl MASO and negative control BAC with GFP expression (E). *SpPKS* (red) and GFP (light blue) transcripts were detected by two-color fluorescent WMISH. DAPI nuclear staining (blue) was performed to facilitate cell counting. Statistical comparisons were made with a two-tailed *T*-test. NSD: no significant difference.

in two independent microinjection experiments. Thus, SpScl rescue requires both correct mosaic incorporation and the presence of BAC DNA encoding SpScl demonstrating specificity of the phenotype to SpScl.

We next investigated the effect of SpScl MASO perturbation on NSM gene expression at the time of peak oral–aboral NSM differentiation (Fig. 6). In SpScl MASO injected embryos, blastocoelar cell specific transcription factors such as *SpGatac*, *SpScl* and *SpEse* are detected throughout the entire NSM territory in the mesenchyme blastula in contrast to their normal oral NSM restriction at this stage (Fig. 6A). Consistent with these results, *SpGatac*, *SpScl* and *SpEse* message prevalence increases moderately in the presence of SpScl MASO (Table S2). In contrast, the expression of pigment cell specific genes, including *SpGcm*, *SpPKS* and *SpCapk* is greatly reduced (Fig. 6B). Notably, in embryos that exhibit reduced expression, these genes are often expressed in a clustered pattern which suggests that local signals may act to maintain the retreating pigment cell domain.

To characterize the temporal influence of *SpScl* perturbation on the expression of pigment cell genes, we examined the time course of *SpGcm* expression in perturbed embryos. In early blastula stage control embryos, *SpGcm* is expressed throughout the NSM ring. Expression disappears from the oral precursors by about 20 hpf but is maintained in the aboral precursors through to pigment cell ingression and differentiation (Ransick and Davidson, 2006). In SpScl MASO perturbed embryos, *SpGcm* is expressed normally at hatched blastula stage throughout the NSM ring. However, *SpGcm* expression is extinguished almost entirely over the course of the following several hours (Fig. 6C and D). Thus *SpGcm* is initially expressed normally in the NSM ring and patterning is disrupted only later at the time of peak localized expression of *SpScl*. Spatial *SpScl* expression is initially evident in sensitive fluorescent WMISH at 18–20 hpf in different regions of the NSM ring (Fig. S2I–L) but at this point expression is very low, transient and variable from embryo to embryo. By approximately 22 hpf, *SpScl* expression is upregulated and localized in the oral NSM (Fig. 1K). SpScl expression may be non-consequential prior to 22–24 hpf, at which time all embryos exhibit homogeneous, localized expression in the oral NSM. If this is the case, the SpScl perturbation time course data are consistent with an SpScl-dependent signal that allows the NSM to proceed with patterning if present and, if absent, in effect recruits other mesoderm into the basal oral NSM program. Alternatively, early expression of *SpScl* in the aboral NSM, though low, may be functional in presumptive pigment cells and perturbation may disrupt an autonomous mechanism.

Regulatory exclusion of SpGatac expression by SpGcm

SpGcm is closely tied to pigment cell differentiation and is one of the earliest transcription factors that is specifically expressed in the NSM (Ransick et al., 2002). Stable and non-variable expression

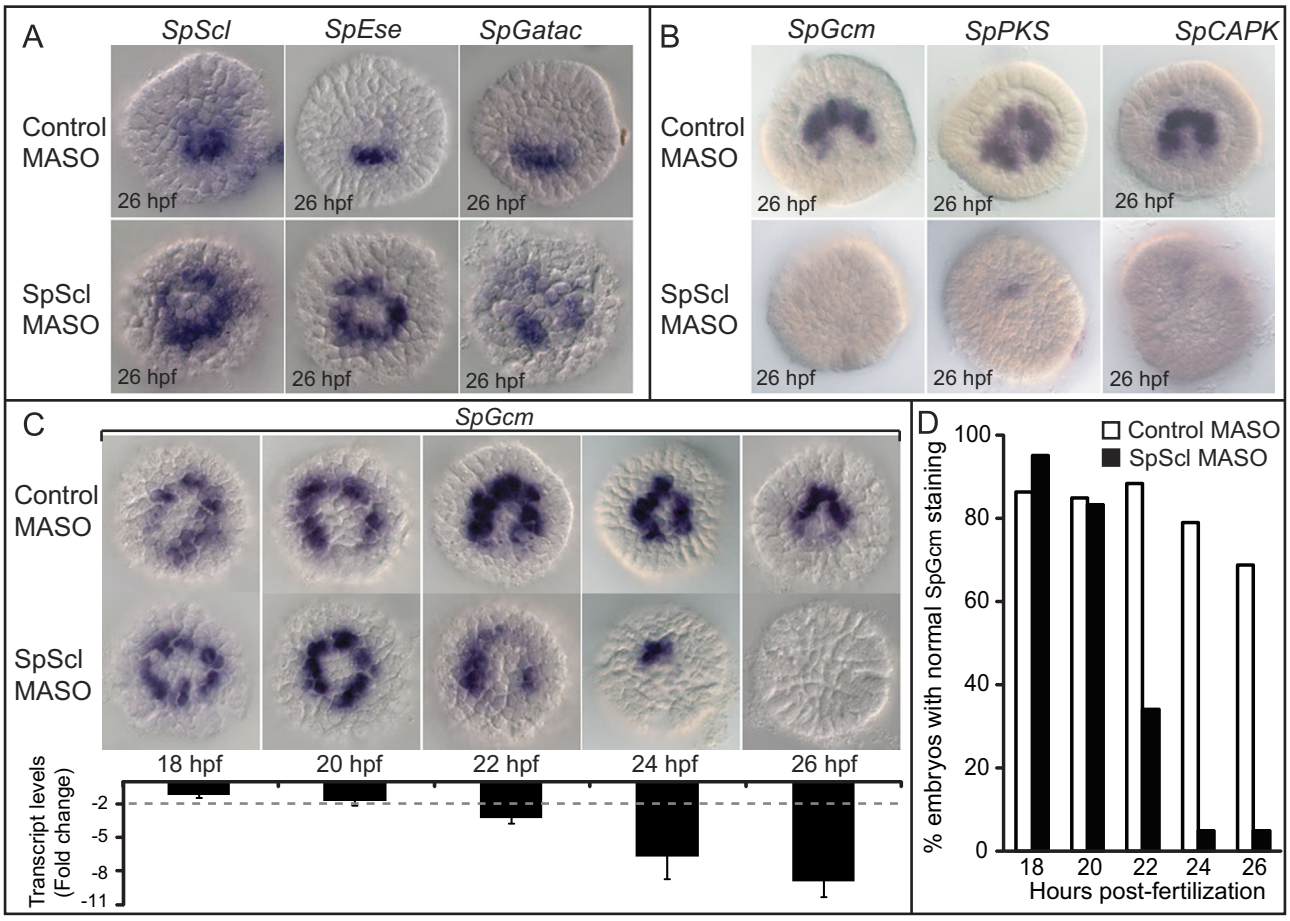


Fig. 6. SpScl perturbation affects transcription of both blastocoelar and pigment cell genes. (A) Blastocoelar cell specific gene expression is expanded in SpScl MASO relative to control MASO injected embryos and is observed in the entire NSM territory, including pigment cell precursors. (B) Pigment cell specific gene expression is limited to a few cells or is undetectable in SpScl MASO embryos compared to control. (C and D) The effect of SpScl perturbation on *SpGcm* expression occurs after establishment of a normal early NSM ring. (C) WMISH time course of *SpGcm* expression in control (top row) and SpScl MASO injected embryos (bottom row), from 18 to 26 hpf, at 2 h intervals. *SpGcm* expression is normal in early SpScl MASO injected embryos, before the regular onset of stable oral *SpScl* expression, but progressively disappears from the pigment cell precursors (22–26 hpf). Fold difference of *SpGcm* transcript levels between control MASO and SpScl MASO injected embryos measured by RT-qPCR is reported below each time point. (D) Percentage of total embryos scored that stained normally for *SpGcm* expression at each time point for control MASO injected (white bar) or SpScl MASO injected (black bar) embryos. A total of 25–35 embryos were scored for each time point and treatment.

of *SpGatac* and *SpScl* is mutually exclusive with *SpGcm* both temporally and spatially. This is particularly true for *SpGatac* where even low expression at early time-points is excluded from cells with high *SpGcm* transcription (Fig. S2A–D). Transcription of the oral NSM transcription factors is elevated only after *SpGcm* is downregulated in this region. In embryos in which SpScl has been perturbed by MASO injection, *SpGcm* downregulation is accompanied by ectopic upregulation of the blastocoelar cell transcription factors in the entire NSM ring (e.g., Fig. 6A). In addition, MASO perturbation of SpGcm results in ectopic expression of *SpGatac* throughout the NSM (Materna et al., 2013; Ransick, personal communication). These observations are consistent with a repressive input from SpGcm into the oral NSM cell regulatory state.

To identify a direct effect of SpGcm on the expression of *SpGatac*, we constructed an *SpGatac*-GFP reporter driven by a DNA fragment that encompasses the 5 kb upstream of the *SpGatac* transcription start site (Fig. 7A). This construct drives expression of GFP in the oral NSM region in a manner similar to the intact 140 kb *SpGatac* BAC-GFP reporter (Fig. S1H–J). We identified five putative binding sites for SpGcm in this region: two proximal sites that are located near the start of transcription (−201 and −216), and three more distal sites (−1930, −3792 and −4363). The proximal sites are conserved in *Lytechinus variegatus*, a sea urchin species that diverged from a common ancestor with *S. purpuratus* approximately 50–100 million years ago (Smith et al., 2006). Two constructs were generated by mutating either the two proximal sites (*5 kb-mut2-GFP*) or all five of the sites (*5 kb-mut5-GFP*; Figs. 7A, S6). The spatial expression of GFP and *SpGcm* was assessed at 25 hpf by two-color fluorescent WMISH. Both constructs produced similar results so analysis of the *5 kb-mut2-GFP* construct is described in detail here. The unmutated 5 kb GFP construct exhibits non-overlapping *GFP*/*SpGcm* expression, with only 11% of embryos (5/44) displaying any co-expressing cells. This is typically seen at the border between oral and aboral NSM territories where *SpGcm* expression appears to be reduced (Fig. 7B). In contrast, 48% of embryos (23/48) transgenic for the *5 kb-mut2-GFP* construct exhibit overlapping expression and GFP message is often observed well into the aboral side of the NSM (Fig. 7C; for the *5 kb-mut5-GFP* construct this number was also 48% [22/46]). Similar results were obtained for independent biological replicates.

Taking into account mosaic expression of the transgene, these data indicate that the mutated form of the reporter is no longer excluded from the SpGcm territory. Mutating the SpGcm sites also increases the proportion of injected embryos expressing GFP, consistent with mosaicism and the hypothesis that expression of *SpGcm* prevents *SpGatac* transcription. Of the embryos injected with the wild type 5 kb GFP construct, 19% ($n=47$) did not express GFP, a higher percentage than observed with the mutant construct (0% [$n=44$] for *5 kb-mut2-GFP*). In an independent replicate

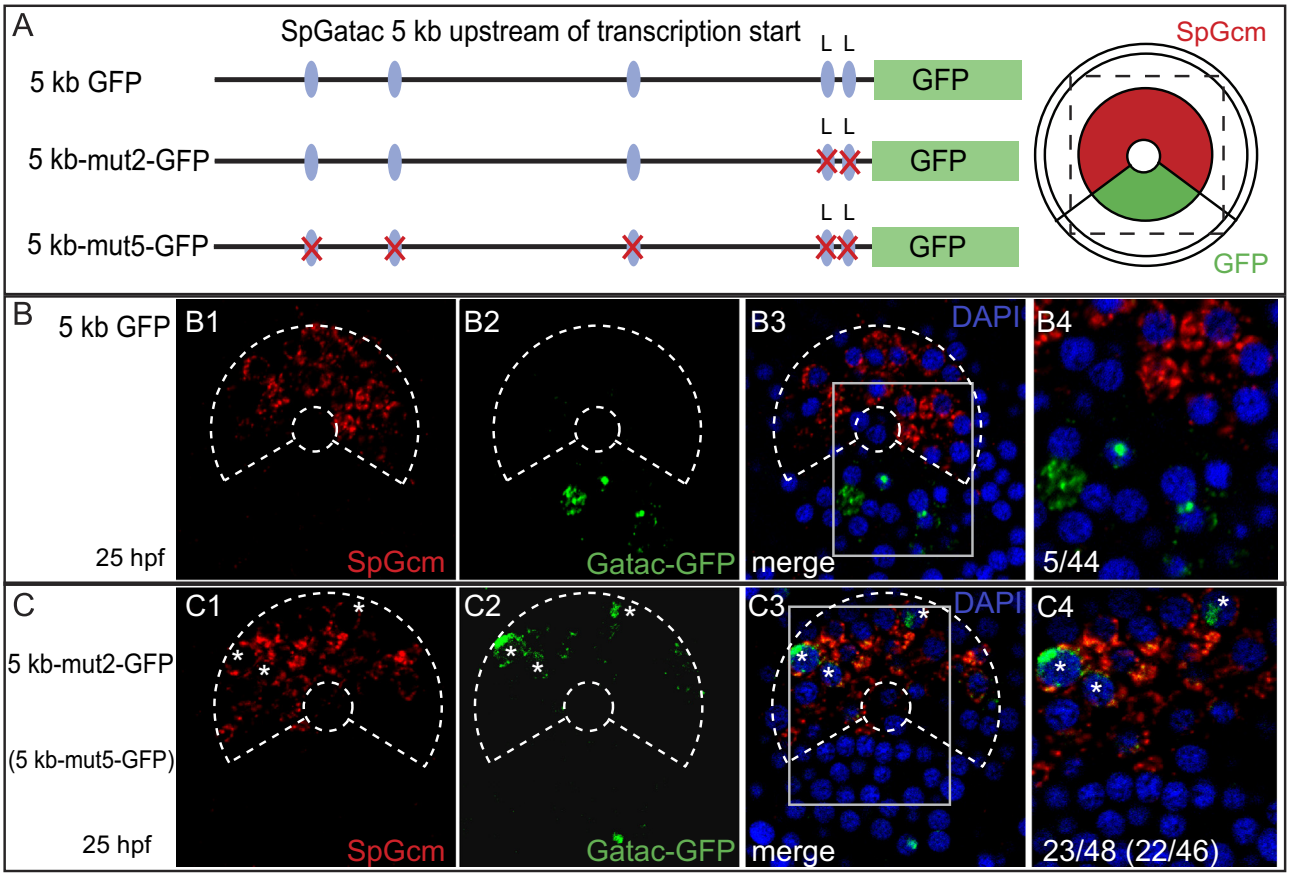


Fig. 7. SpGcm represses expression of *SpGatac* in the presumptive pigment cells. (A) Schematic representation of a 5 kb *SpGatac*-GFP reporter construct. The position of putative SpGcm binding sequences is represented by blue ovals, red crosses indicate the sites mutated in the *5 kb-mut2-GFP* and *5 kb-mut5-GFP* constructs. L, sites found conserved in *L. variegatus*. Right: Diagram of a mesenchyme blastula stage embryo showing the location of pigment cell precursors expressing *SpGcm* in red and blastocoelar cell precursors expressing *GFP* under the control of the reporter in green. (B and C) Apotome fluorescent images of WMISH of mesenchyme blastula stage embryos with probes for *SpGcm* (B1, C1) and *GFP* (B2, C2). Merged images (B3, C3), including DAPI nuclear staining (blue), show the exclusion or overlap between both probes, with insets enlarged in B4 and C4 for clearer comparison. A representative image of the most prevalent phenotype is shown for each reporter. (B) The majority of embryos injected with the wild type reporter display no overlap between the *GFP* and *SpGcm* staining. The minority that do (5/44) exhibit overlap at the border between oral and aboral NSM. (C) Embryos injected with the *5 kb-mut2* or *5 kb-mut5* reporters exhibit an increased frequency of overlap between *GFP* and *SpGcm* staining, (23/48 and 22/46, respectively) that is often well into the aboral NSM. Dotted white outline defines the region occupied by the pigment cell precursors; asterisk indicates cells expressing both *SpGcm* and *GFP*.

experiment, proportions of 38% (6/16) for wild type and 6% (1/16) for the mutant construct were observed. Notably when these embryos were followed at later time points, expression of GFP from the mutant *SpGatac* reporters was regularly evident in differentiated pigment cells (Fig. S6).

Discussion

A transcription factor tool kit for immunocyte specification

We have characterized the downstream function of two regulators of immune cell specification in the sea urchin, SpGatac and SpScl. These have well-known counterparts in vertebrate hematopoiesis but their orthologs have not yet been implicated in immune cell development in invertebrates. Notably, in both sea urchins and vertebrates, homologs of Gata-1/2/3 and Scl are expressed at the first stages of immunocyte development. In vertebrates, Gata-1 and Gata-2 bind DNA both independently and cooperatively in complexes that include heterodimers of Scl and the E proteins E2A and HEB (Lecuyer and Hoang, 2004; Massari and Murre, 2000). GATA-Scl complexes are bridged by Lmo2 (Matthews and Visvader, 2003). Therefore, in the sea urchin co-expressed SpGatac and SpScl likely co-regulate an overlapping subset of target genes as a cooperative complex in the oral NSM. This complex likely includes SpE-protein and SpLmo2. In vertebrate systems, the transcriptional activity of Gata-1 is mediated by the presence of Scl such that sites occupied by Gata-1–Scl/E2A complexes confer positive regulation, whereas sites bound by Gata-1 alone confer repression (Tripic et al., 2008). Similarly, in the sea urchin genes may be differentially regulated by SpGatac–SpScl complexes and sites bound independently by SpGatac. Given the differing phenotypes that result from perturbing SpGatac or SpScl function individually, it is difficult at present to analyze potential co-regulation by these factors. Co-injection of both SpGatac and SpScl morpholinos results in a severe gastrulation arrest (CMS and JPR, data not shown) that is difficult to interpret in a purely phenotypic context. As direct target genes are identified for each of these regulators, identification of co-regulated targets will become more feasible from a cis-regulatory standpoint.

Early oral *SpGatac* and *SpScl* expression prefigures the site of presumptive blastocoelar cell development. This is evident in WMISH and in the expression of BAC-based reporter transgenes in which GFP expression persists in blastocoelar cells after ingression. It is also consistent with the downregulation of the blastocoelar cell immune marker *SpMacpfA2* upon SpGatac perturbation. *SpGatac* and *SpScl* are later activated in the coelomic pouch cells (as well as in the aboral skeletal mesenchyme cells) but this represents a separate phase of *SpGatac* expression. Although

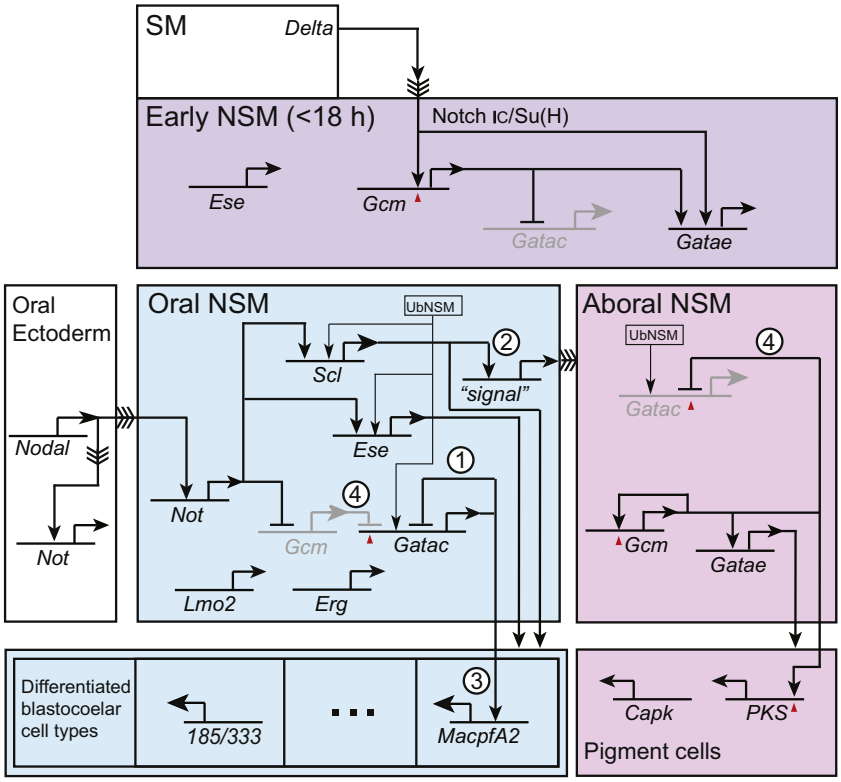


Fig. 8. Regulatory interactions in the progression to oral NSM differentiation. The downstream functions of SpGatac and SpScl presented here are integrated with those from several recent papers investigating the establishment of the NSM and its subsequent oral–aboral patterning (Duboc et al., 2010; Materna and Davidson, 2012; Materna et al., 2010, 2013; Ransick and Davidson, 2006, 2012). The early NSM is established with *SpGcm* expression in response to an SM Delta signal. Spatially restricted expression of the transcription factor *SpEse*, which eventually is expressed exclusively in the oral NSM, is initially expressed throughout the NSM ring just after initiation of *SpGcm* expression. *SpGatac* and *SpScl* are expressed throughout the NSM after 18 hpf but expression is spatially variable and low. In the later blastula *SpGcm* is downregulated in the oral NSM. This allows the expression of *SpGatac* in the oral NSM. Since *SpGatac* and other oral NSM transcription factors can be expressed throughout the NSM in the absence of *SpGcm* the presence of NSM activator or activators is designated (UbNSM). A negative auto-regulatory feedback loop of *SpGatac* onto itself is also present which may play a role in downregulating *SpGatac* expression in the late gastrula stage (1). A SpScl-dependent signaling mechanism which may represent a true signal or a downstream effect of general vegetal plate disruption is responsible for the stability of the oral–aboral division of the NSM and the maintenance of the aboral pigment cell program in the late blastula (2). SpGatac is also essential for expression of a downstream differentiation gene in an oral NSM derived immune cell subtype (*SpMacpfA2*) (3). SpGcm can directly repress *SpGatac* in the aboral NSM (4). Red triangles indicate inputs validated by cis-regulatory analysis other inputs may be direct or indirect.

coelomic pouch cells also arise from the NSM (Ruffins and Ettensohn, 1996), we find no clear evidence of maintenance of high expression in potential precursors in the late gastrula once the blastocoelar cells have ingressed, suggesting that expression in the coelomic pouches is reinitiated later or may emerge from low expressing cells.

The transient expression of *SpGatac* and *SpScl* at high levels in the oral mesoderm overlaps the developmental period during which the regulatory state of this region begins to diverge from that of the aboral pigment cell precursors. These cells express differentiation markers starting at blastula stage and undergo migration early in gastrulation. SpGatac and SpScl may have a role in repressing parallel differentiation processes in the oral NSM. Unlike the aboral pigment cell precursors, we observe expression of differentiation markers only very late in gastrulation as the blastocoelar cells migrate from the archenteron tip after *SpGatac* and *SpScl* expression is downregulated (e.g., *SpMacpfA2* and the immune effector *Sp185/333*, unpublished). Despite much effort we have been unable to identify any markers of blastocoelar cell differentiation that are expressed prior to ingression, in sharp contrast to markers for pigment cell differentiation. This potential function for SpGatac and SpScl in maintaining an undifferentiated state is consistent with the observed multipotency of the blastocoelar cell precursors (Ettensohn et al., 2007). The expansion of blastocoelar cell regulators into the aboral NSM when SpScl is perturbed in experiments described here may relate to a role for this transcription factor in the re-emergence of an undifferentiated blastocoelar cell program when the normal blastocoelar cell specification is disrupted. Notably emergent expression of *SpScl* is seen also in transfating endoderm when mesodermal cells are experimentally ablated (Sharma and Ettensohn, 2011). Thus there is reason to speculate that, as in vertebrates, the co-expression of GATA and Scl homologs in the oral NSM may coincide with a system for maintaining developmental plasticity.

A foundation for characterizing the GRN that governs larval immunocyte development

The sea urchin larva is equipped with a complex immune system mediated by several types of immunocytes. With the exception of pigment cells, these arise from the oral NSM. Although *SpGcm* is expressed early in all NSM cells, this factor appears to be closely tied to pigment cell differentiation (Ransick and Davidson, 2006). Its expression is not strictly prerequisite for activation of the blastocoelar cell transcription factors since *SpGatac* is activated in SpGcm MASO injected embryos (Materna et al., 2013). As with the larval skeleton, embryonic pigment cell development may represent a precocious differentiation program to rapidly deploy functional pigment cells in the embryo. The basal program for immunocyte development may lie in the oral NSM and in the network surrounding *SpGatac* and *SpScl*. This is consistent with the expression of *Gatac* and other oral mesoderm transcription factors characterized in a sea cucumber and starfish (McCauley et al., 2012).

These are integrated with published findings for the establishment of oral–aboral NSM transcription factor patterning (Duboc et al., 2010; Materna and Davidson, 2012; Materna et al., 2010, 2013) and pigment cell specification (Ransick and Davidson, 2006, 2012) in Fig. 8. Four interactions are evident from the data presented here. (1) SpGatac negatively affects expression of its own transcript and this may contribute to the later downregulation of *SpGatac* at gastrulation. Determining whether this feedback is direct or indirect requires further regulatory analysis. (2) Expression of *SpScl* in the oral NSM may be required for oral–aboral NSM patterning to proceed beyond the mesenchyme blastula stage. The breakdown of NSM patterning in the absence of SpScl and the ability to rescue this phenotype with a small patch of transgenic SpScl expression suggests the presence of an SpScl-dependent signal that is required for normal oral–aboral specification. In any event, these experiments demonstrate the plasticity of the NSM even after pigment cell differentiation markers are strongly expressed. (3) Perturbation of SpGatac negatively affects downstream expression of an immune marker during blastocoelar cell differentiation. (4) At least part of the presumptive blastocoelar program, namely *SpGatac* expression, is repressed by SpGcm in differentiating pigment cells.

Notably the activity of the single sea urchin E protein bHLH factor, SpE-protein, is likely to be differentially modulated between the oral and aboral NSM compartments by several mechanisms in this process. These include the formation of different SpE-protein regulatory complexes in partnering with SpScl in the oral region and transiently with elevated SpId in the aboral region and alternative splicing of SpE-protein itself that is differential across the oral-aboral NSM (C.M.S., C.S.S. and J.P.R., unpublished).

Conserved regulators of immunocyte specification

The sea urchin homolog of the vertebrate hematopoietic GATA factors (Gata-1, -2, -3) was first isolated from adult coelomocytes (Pancer et al., 1999), where it is expressed in the phagocytic subset of these cells. A role in the development of immune cells in the embryo is evident here in its function upstream of an immune effector gene (*SpMacpfA2*). Another invertebrate GATA factor, *Drosophila Serpent* (*srp*), is a regulator of hemocyte development, but is not orthologous to the hematopoietic clade of vertebrate GATA genes (Gillis et al., 2008). The *Drosophila* GATA family is expanded, however, and it is possible that the function for an ancient GATA factor in immunocyte development was co-opted by this divergent ortholog in the fly. The *Drosophila* ortholog of vertebrate *Gata-1/2/3* (*Grain*) has not been reported to regulate hemocyte specification, although it is a potential regulator of antibacterial peptides in the midgut (Senger et al., 2006). In *Platynereis dumerilli*, a polychaete annelid, expression of the single *Gata-1/2/3* ortholog is restricted to ectoderm during early development and does not appear to function in mesoderm where a *Gata-4/5/6* ortholog is expressed (Gillis et al., 2007). Our findings suggest that a role of *Gata-1/2/3* orthologs in immunocyte development extends at least to the common ancestor of living deuterostomes.

The vertebrate homolog of *SpScl* is a key early regulator of hematopoietic stem cell development (Lacombe et al., 2010; Souroullas et al., 2009) and also functions in the differentiation of mast cells (Salmon et al., 2007), megakaryocytes (Gekas et al., 2009) and erythrocytes (Schuh et al., 2005). As with the *Gata-1/2/3* paralog group, all of the mouse *Scl* paralogs, *Scl*, *Tal-2* and *Lyl-1*, participate in hematopoietic processes (Capron et al., 2006; Gekas et al., 2009) suggesting an association with immunity that dates at least to gene duplication in the ancient vertebrate ancestor. Although no role has been identified for the *Drosophila Scl* homolog in regulating hemocyte development or function, an *Scl* homolog is expressed in the hemocytes of the Pacific oyster, *Crassostrea gigas* (Barreau-Roumiguiere et al., 2003). Thus a function for Scl in hemocyte development may extend to the common bilaterian ancestor.

Several additional transcription regulators that are expressed in sea urchin coelomocytes and blastocoelar cells are homologs of factors that are well known to form complexes with Scl and Gata-1/2/3 in vertebrate hematopoiesis, potentially indicating a wider conserved network. Fli-1 is an ETS family transcription factor that interacts in a regulatory circuit with Gata-2 and Scl that is critical for maintaining a multipotent state in hematopoietic stem cells (Pimanda et al., 2007). *SpErg*, the single ortholog of vertebrate *Fli-1* and *Erg* (Qi et al., 1992), is expressed in the mesoderm at mesenchyme blastula stage (Rizzo et al., 2006). Within the NSM, *SpErg* is orally localized and later it is expressed in the coelomic pouches and aboral skeletal cells as are *SpGatac* and *SpScl* (Fig. S3C–F), suggesting that interactions among these transcription factors may be conserved. Remarkably, the transient co-expression of *SpGatac*, *SpScl* and *SpErg* in the small patch of presumptive blastocoelar cells parallels the orthologous triad of transcription factors (*Gata-2*, *Scl* and *Fli-1*) that maintain an undifferentiated state in hematopoietic stem cells in vertebrates (Pimanda et al., 2007).

Further regulatory similarity is evident between sea urchin and vertebrate immune cells. PU.1 is an ETS family regulator of both myeloid and lymphoid hematopoiesis in vertebrates (Singh et al., 1999). Expression of *SpPU.1*, an ortholog of vertebrate *PU.1*, *SpiB* and *SpiC*, is absent from the early embryo but later expression is observed in gut-associated blastocoelar cells of the feeding larva (Rizzo et al., 2006). A role for ESE in vertebrate hematopoiesis has not been reported, however, its expression is associated with endothelial immunity and monocyte cell function in inflammation (Grall et al., 2005; Rudders et al., 2001). In addition, an ortholog of *Ese* is expressed in the hemocytes of the sea scallop (*Chlamys farreri*; Ma et al., 2009) so expression of *SpEse* in the NSM may have immune function. Notably, all of these ETS factors (*SpPU.1*, *SpErg* and *SpEse*) are co-expressed also in adult sea urchin coelomocytes (Fig. S7).

Finally, the sea urchin embryonic mesoderm also shares factors that have only been associated with immune cell differentiation in protostome invertebrates. For instance, in the sea urchin, SpGcm is a critical factor for pigment cell differentiation in the embryo (Calestani et al., 2003; Ransick and Davidson, 2006; Ransick et al., 2002) and is expressed in the red spherule cells of adult coelomocytes. A homolog of *SpGcm* is well characterized as a regulator of *Drosophila* plasmatocyte development (Bernardoni et al., 1997; Lebestky et al., 2000). An ancient function for Gcm in bilaterian immunity may have been lost in the vertebrate lineage or conversely its usage in immune cell development in Diptera and the sea urchin may be independently derived.

Conclusions

The findings presented here are focused on the downstream consequences of perturbation of SpGatac and SpScl to begin to elucidate their roles in the sub-specification of the NSM. We identify several regulatory connections which, in combination with published studies focused on the establishment and oral–aboral partitioning of the NSM, begin to shed light on the network that eventually leads to the segregation and subdivision of the oral NSM and the differentiation of immune blastocoelar cell-types. We also introduce a BAC-based method for phenotypic rescue that will be more widely applicable. Notable similarities are evident in the contexts in which SpGatac and SpScl function in the differentiation of the oral NSM derivatives and in the function of their vertebrate

homologs in the course of immune cell development. The extent and depth of this similarity will be evident as more detailed gene regulatory network models involving these transcription factors are constructed.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.06.019.

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